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ORIGINAL REPORT

INHIBITORY ACTION OF PHA ON THE APPEARANCE OF THYMIC *RETICULAR CELLS IN VITRO*

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INTRODUCTION

Phytohemagglutinin (PHA) is well known to stimulate the division of lymphocytes *in vitro*, especially thymus-derived ones¹⁾. Despite of the observation by Ioachim²⁾ that PHA stimulates mitosis in a variety of cultured cell lines, others have reported an inhibitory effect of PHA on DNA synthesis in cultured human³⁾ and mouse⁴⁾ cells. The action of PHA on mammalian cells other than lymphocytes seems to have been studied insufficiently both *in vivo* and *in vitro*.

This work deals with the inhibitory action of PHA on thymus cells *in vitro*, the mechanism of inhibition being analyzed at the cellular level.

MATERIALS AND METHODS

1) PHA-P (Difco Laboratories, Detroit, Mich.) and a crude PHA preparation extracted from kidney beans by the method of Rigas and Osgood⁵⁾ were used. Their protein concentrations were estimated by the micro-Kjeldahl method⁶⁾.

2) Cultivation of rat thymus cells.

(a) Primary culture.

Thymuses were removed from 2 to 3 adult Sprague Dawley rats and teased in a heparinized cold Hanks solution the resulting cell suspension being allowed to stand for 10 minutes. Floating cells used for the culture consisted of about 95% small lymphocytes and 5% medium- and large-sized mononuclear cells. About 2×10^6 /ml cells were inoculated in RPMI medium 1640 supplemented with 30% fetal calf serum (GIBCO, New York, N.Y.) with 50 units of penicillin

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and 50 μg of streptomycin per ml. The cultivation was carried out in small Petri dishes or Leighton tubes in 5% CO_2 in air at 37°C.

(b) Long-term culture of *reticular cell-like cells* (RCL-cells)

On the 5th day of the primary culture, floating lymphocytes were decanted and round cells adhering to the glass were maintained for 10 days. On the 8th to 10th day of the primary culture the cells showed a distinct morphological character: they displayed large cytoplasm with fine fiber-like structures and a large nucleus with distinctive nucleoli. They were freed from the glass by 0.1% trypsin treatment for subculture.

3) Cell labeling

RCL-cells which had been maintained for 2 to 5 months were labeled with ^3H -thymidine (1 $\mu\text{Ci/ml}$)(^3H -TdR), ^3H -uridine (5 $\mu\text{Ci/ml}$)(^3H -UdR) or ^3H -leucin (5 $\mu\text{Ci/ml}$)(^3H -Leu) for 1 hr. After collecting and washing with cold saline, the cells were treated with 5% perchloric acid. Then the cell pellet obtained was dissolved in a 0.2 M KOH solution. The radioactivity was measured in a liquid scintillation counter (Horiba Co., Tokyo, Japan). Autoradiography was done by the dipping method, using SAKURA NR-M2 emulsion (Konishiroku Photo Ind. Co. Otd., Tokyo, Japan).

RESULTS

1) Effect of PHA on primary cultures

At 2 to 4 days of primary cultivation of normal rat thymus cells, round cells, much larger than small lymphocytes, appeared at the bottom of the dish in addition to floating small cells (Fig. 1). Thereafter, the cells transformed gradually into RCL-cells. The cytoplasm of the RCL-cells flattened and spread on the glass, sometimes being associated with fine fiber-like structures, their nuclei becoming larger (Fig. 2). When 60 $\mu\text{g/ml}$ of PHA-P was added to the culture at the beginning of cultivation, neither glass-adherent round cells nor the subsequent occurrence of RCL-cells was observed. On the other hand, extensive aggregation of floating cells was noted. Sixty $\mu\text{g/ml}$ of PHA was equivalent to 5 μl PHA-P (Difco)/ml which was reported by Peavy *et al.*⁷⁾ to be the optimal dose for its mitogenic activity on mouse spleen cells. The crude PHA showed a similar inhibitory action on thymus cells in the same manner.

The dose and time effects of the PHA treatment have been studied in triplicate culture and the results obtained are shown in Fig. 3. The inhibitory action of PHA was dependent on both the dose and the time. A high dose (60 $\mu\text{g/ml}$) of PHA was fully effective and complete or nearly complete inhibition was seen even on the 3rd day of cultivation. However, a low dose (6 μg , or 0.6 $\mu\text{g/ml}$) resulted in only a partial suppression on days 0 and 1. The inhibition was no longer observed on the 5th day even at the highest dose of PHA. These inhibitions were confirmed repeatedly in 3 successive experiments.

2) Effect of PHA-P on isolated RCL-cell cultures

RCL-cells, isolated and serially transplanted *in vitro* were treated with PHA-P (60 $\mu\text{g/ml}$) during the 5th to 20th generation. PHA-P was added to the cell cultures under two different conditions; the dissociated and the glass adherent monolayer. No morphological difference between these two conditions was observed and the treated cells underwent cell division normally

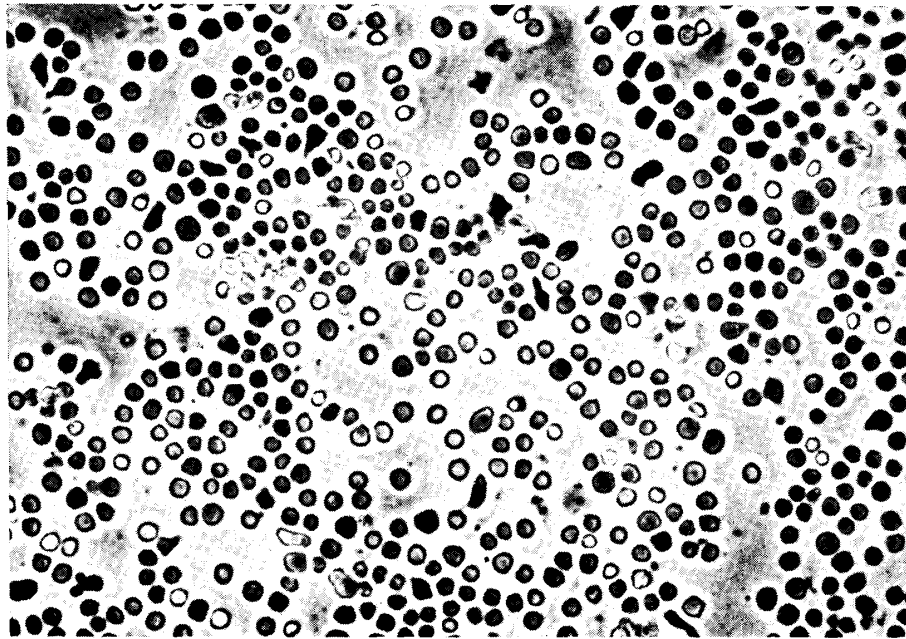


Fig. 1 Primary thymus cell culture observed by phase contrast microscopy 2 days after inoculation. (357×354)

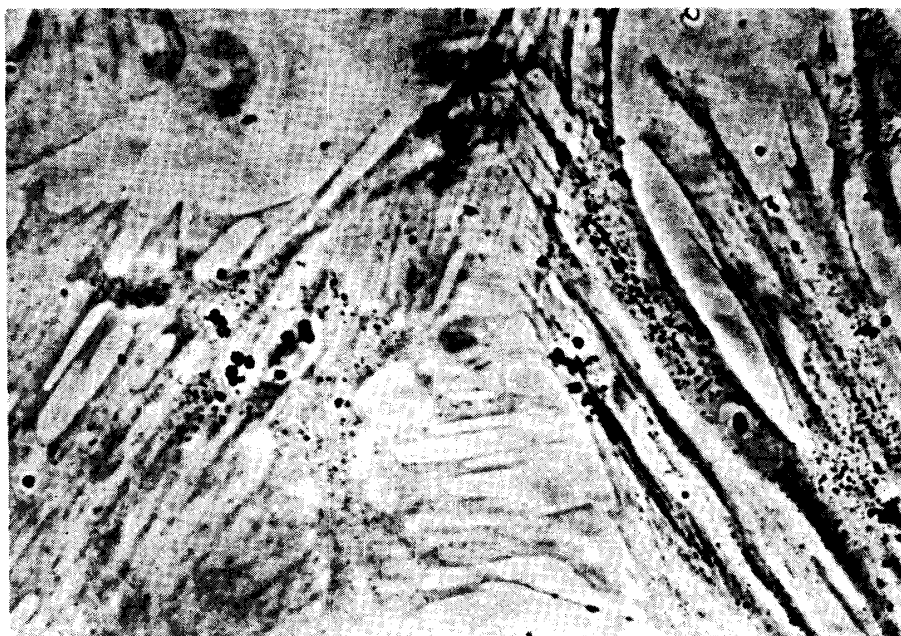


Fig. 2 RCL-cells by phase contrast microscopy at 10 days after culture. (357×354)

throughout 7 days. On the 4th day of PHA administration, the cells were labeled with H^3 -UdR and H^3 -Leu ($5 \mu\text{Ci/ml}$ each) for 1 hr. As shown in Table 1, no significant decrease in either RNA or protein synthesis was observed. An autoradiographic study of the incorporation of ^3H -TdR ($0.5 \mu\text{Ci/ml}$, for 3 hours) showed that mean labeling indices were 6.0% in the control and 5.4% in the experimental. Therefore, it may be said that PHA-P has no inhibitory effect on established RCL-cell cultures.

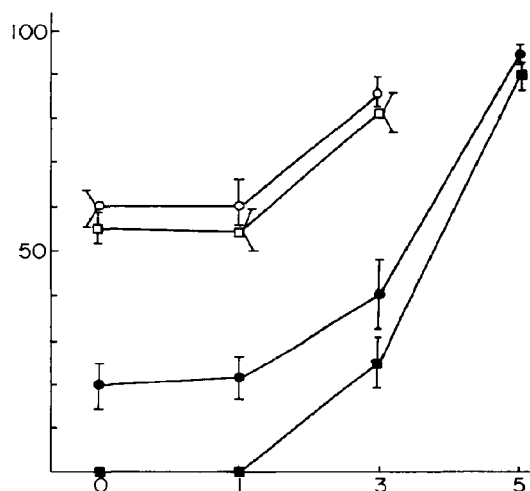


Fig. 3 Dose and time effects of PHA-P on the occurrence of RCL-cells.
Abscissa: Time in days of PHA-P administration after inoculation of thymus cells.

Ordinate: Mean percent frequency of RCL-cell occurrence compared with that of the control culture.

Mean number of round cells to be RCL-cells later in control tubes was 738 per 2×10^6 cells/tube on the 6th day.

PHA-P 0.6 µg/ml (○—○), 6 µg/ml (□—□), 20 µg/ml (●—●), 60 µg/ml (■—■).

Each vertical bar shows standard deviation.

Table. 1 Effect of PHA-P on RNA and Protein Synthesis of RCL-cells.^{a)}

	H ³ -UdR	H ³ -Leu
Control ^{b)}	12077.5	15360.3
PHA-P-treated ^{c)}	11607.3	13251.6
Lymphocyte-added ^{d)}	10480.2	13125.2

^{a)} 3×10^5 RLC-cells in a Petri dish were treated with PHA-P or PHA-P plus fresh thymus cells. Incorporation of labeled precursors was measured as cpm/culture at 4 day of culture. Each value represents the average of three cultures.

^{b)} Cultivation was done without addition of PHA.

^{c)} PHA-P (60 µg/ml) was added.

^{d)} 4×10^6 PHA-treated thymus cells were added in the culture. After the floating lymphocytes were decanted, H³-UdR or H³-Leu was added.

3) Effects of thymic lymphocytes on isolated RCL-cells

It was possible that the inhibitory effect of PHA on primitive RCL-cells in primary culture was brought about by an aggression of PHA-activated thymic lymphocytes against allogeneic reticular cells, as suggested by others^{8,9)}. The following experiments were done in order to test this possibility. The RCL-cells, suspended or monolayered, were mixed with freshly prepared rat thymus cells. Four million thymus cells were added to 2×10^4 or 2×10^5 RCL-cells. During the first 6 days of the mixed cultures with or without PHA-P, no morphological

change was found in the RCL-cells. RNA and protein synthesis of the RCL-cells treated simultaneously with both PHA-P and thymus cells was not significantly different from that of RCL-cells treated only with PHA, so far as the isotope incorporation on the 4th day of treatment is concerned (Table 1). Therefore, the postulation is unlikely that PHA-activated thymic lymphocytes affected the RCL-cells, causing the inhibitory action of PHA in the primary culture of the thymus.

DISCUSSION

It has been demonstrated in the present experiments that PHA has a suppressive effect on RCL-cell formation in cultured rat thymus. This effect was observed only in cells at initial stages of development in primary culture. When PHA was added to RCL-cell cultures isolated from primary cultures and maintained up to 6 months, the inhibitory effect was no longer seen even at the initial stage of subculture.

Holm and Pearlmann⁸⁾ reported that human lymphocytes treated with PHA were more cytotoxic to target tumor cells than were normal lymphocytes. Knight and Thorbecke⁹⁾ observed that rat thymocytes act as killer-cells in the mixed lymphocyte reaction in appropriate conditions. Therefore the possible participation of PHA-activated lymphocytes in the growth suppression of RCL-cells in the primary culture was tested, for Sprague Dawley rats used in these experiments were not highly inbred and an allogeneic reaction of PHA-stimulated thymic lymphocytes against the RCL-cells might occur in these experimental systems. Data from our experiments demonstrated that thymic lymphocytes treated with PHA were not aggressive against the RCL-cells under the conditions described.

Han¹⁰⁾ suggested that primitive reticular cells, round mononuclear cells may differentiate into phagocytic reticular cells and reticular cells with fibers. The present experiments seems to support this observation. PHA may act directly on the large mononuclear cells in the primary culture to inhibit their further development to RCL-cells. PHA is no longer toxic after this transformation.

SUMMARY

When cultured rat thymus cells were treated with PHA at the time of plating, reticular cell-like cells which are regular elements of cultured thymus did not appear. However, PHA added to primary cultures on the 3rd or 5th day showed no inhibitory effect on such reticular cell-like cells. In addition it was not cytotoxic to the established reticular cell-like cells which were isolated and maintained *in vitro* for six months. Thymic lymphocytes activated by PHA were not aggressive against such cells. This phenomenon may be due to the selective toxicity of PHA to large round cells which are precursors of the reticular cell-like cells.

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REFERENCES

- 1) Rodey, G. E. and Good, R. A.: The in vitro response to phytohemagglutinin of lymphoid cells from normal and neonatally thymectomized adult mice, *Int. Arch. Allergy*, 36: 399, 1969.
- 2) Ioachim, H. L.: Effects of phytohaemagglutinin in vitro on cells other than lymphocytes, *Nature*, 210: 919, 1966.
- 3) Caso, L. V.: The effects of phytohemagglutinin on the growth and histochemical properties of mammalian cells in tissue culture, *Anat. Rec.*, 162: 495, 1968.
- 4) Dent, P. B.: Inhibition by phytohemagglutinin of DNA synthesis in cultured mouse lymphomas, *J. Nat. Cancer Inst.*, 46: 763, 1971.
- 5) Rigas, D. A. and Osgood, E. E.: Purification and Properties of the phytohemagglutinin of *Phaseolus vulgaris*, *J. Biol. Chem.*, 212: 607, 1955.
- 6) Koch, F. C. and McMeekin, T. L.: A new direct nesslerisation micro-Kjeldahl method and a modification of the Nessler-Folin reagent for ammonia, *J. Amer. Chem. Soc.*, 46: 2066, 1942.
- 7) Peavy, D. L., Adler, W. H. and Smith, R. T.: The mitogenic effects of endotoxin and staphylococcal enterotoxin B on mouse spleen cells and human peripheral lymphocytes, *J. Immunol.*, 105: 1453, 1970.
- 8) Holm, G. and Pearlmann, P.: Quantative studies on phytohaemagglutinin-induced cytotoxicity by human lymphocytes against homologous cells in tissue culture, *Immunology*, 12: 525, 1967.
- 9) Knight, S. C. and Thorbecke, G. J.: Ontogeny of cellular immunity: Development in rat thymocytes of mixed lymphocyte reactivity to allogeneic and xenogeneic cells, *Cellular Immunol.*, 2: 91, 1971.
- 10) Han, S. S.: The ultrastructure of the mesenteric lymph node of the rat, *Amer. J. Anat.*, 109: 183, 1961.